

CHARACTERIZATION AND THERMOSTABILITY STUDY OF  
INVERTASE BY *ASPERGILLUS NIGER* IN SUBMERGED  
CULTURE

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Thesis submitted in fulfillment of the requirements for the award of  
Degree of Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical Engineering and Natural Resources

UNIVERSITI MALAYSIA PAHANG

FEBRUARY 2013

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## ABSTRACT

Invertase is a commercially important enzyme used for hydrolysis of sucrose. The hydrolysis of sucrose yields a mixture of glucose and fructose, or famous as name invert syrup, this enzyme was widely used in food and beverage industries. Objectives of this research are to study the pH and thermostability of invertase by *Aspergillus niger*, to study the enzyme kinetics of invertase and to study the effect of sucrose concentration during incubation towards invertase activity. *A. niger* produced high levels of invertase under culture conditions (potato dextrose agar) on fourth day of incubation at an optimum temperature 30°C, inoculum at 30°C at 250 rpm and using sucrose as a substrate by submerged fermentation (SmF) also with same culture conditions. Separation between enzyme and other cell was done by centrifugation and continued with study the pH and thermostability of enzyme by terminated by heating. Thermostability of enzyme were investigated by determining the enzymatic reaction subjected to 40°C, 45°C, 50°C, 55°C and 60°C. Michaelis-Menten parameters ( $V_{\max}$  and  $K_m$ ) were determined for four different sucrose concentration which are 0.2, 0.4, 0.6, 0.8 g/L. Result suggested that optimum pH is 5.0 and temperature is 55°C. While both  $V_{\max}$  and  $K_m$  would increase at higher temperatures because temperature will alter the shape of enzyme by changing its ionic form at active site.

## ABSTRAK

Invertase adalah enzim komersial penting yang digunakan untuk hidrolisis sukrosa. Proses hidrolisis sukrosa menghasilkan campuran glukosa dan fruktosa, atau terkenal sebagai nama terbalikkan sirap, enzim ini telah digunakan secara meluas dalam industri makanan dan minuman. Objektif kajian ini adalah untuk mengkaji kestabilan pH dan suhu invertase, untuk mengkaji enzim kinetik invertase dan untuk mengkaji kesan kepekatan sukrosa semasa inkubasi terhadap aktiviti invertase. *Aspergillus niger* menghasilkan invertase pada tahap tinggi bawah keadaan kultur (kentang agar dekstroza) pada hari keempat pada suhu optima 30°C, inokulum 30°C pada 250 rotasi per minit dan menggunakan sukrosa sebagai substrat oleh kultur tenggelam juga dengan keadaan kultur yang sama. Pengasingan antara enzim dan sel lain telah dilakukan melalui proses pemusingan dan teruskan dengan kajian kestabilan pH dan suhu enzim diakhiri dengan pemanasan. Kestabilan suhu enzim telah dinilai dengan menentukan tindak balas enzim terhadap 40°C, 45°C, 50°C, 55°C dan 60°C. Michaelis-Menten parameter ( $V_{max}$  dan  $K_m$ ) telah ditentukan oleh empat kepekatan sukrosa 0.2 g/L, 0.4 g/L, 0.6 g/L dan 0.8 g/L. Keputusan mencadangkan bahawa pH optima ialah 5.0 dan suhu optima adalah 55°C. Walaupun kedua-dua  $V_{max}$  dan  $K_m$  akan meningkat pada suhu yang lebih tinggi kerana suhu akan mengubah bentuk enzim dengan menukar bentuk ionik di tapak aktif.

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## LIST OF SYMBOL / ABBREVIATIONS

HFS	High Fructose Syrup
$K_m$	Maximum Reaction Rate or substrate concentration when the enzyme has half-maximal velocity
$V_{max}$	Maximum Velocity
GRAS	Generally Recognized as Safe
S	Substrate
E	Enzyme
ES	Intermediate complex
MARDI	Malaysian Agricultured Research and Development Institute
PDA	Potato Dextrose Agar
HPLC	High-Performance Liquid Chromatography
$(NH_4)_2SO_4$	Ammonium Sulphate
$FeSO_4$	Iron Sulphate
$MgSO_4 \cdot 7H_2O$	Magnesium Sulfate Heptahydrate
rpm	Revolutions per minute
ppm	Parts per million
SmF	Submerged Fermentation
SSF	Solid State Fermentation
IU/mL	Concentration of one international unit per one millilitre of system volume

## CHAPTER 1

### INTRODUCTION

#### 1.1 Background of Proposed Study

Enzymes are proteins that produced by all living cells as catalysts for specific chemical reactions. In enzymatic reactions, the molecule at beginning is called substrates, and then they convert into different molecules called products. Almost all biological cells need enzymes to increase the rate of chemical reactions in order to occur at rates sufficient for life. Enzymes are very important part in some food processes, such as making of cheese, bread, wine and beer, for thousands of years (Dewdney, 1973). Invertase (invert sugar) is one of the most widely used enzymes in confectionery industry for preparation of jams and candies (Klein *et al.* 1989). Invertase or  $\beta$ -fructofuranoside catalyzes the cleavage of sucrose to glucose and fructose. Invertase usually used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup (HFS).

Invertase must be one of the best known of all enzyme systems because of its historical importance, but only recently have its properties been subjected to rigorous examination. Recently, many research studied about enzyme kinetics on invertase activity in dilute solution (Uma *et al.*, 2012). More recently there has been an increment of interest in its catalytic properties at higher concentrations of substrate and products because of commercial applications in the production of liquid, free sugar solutions. This study has good potential for food biotechnological applications in increasing biotechnological importance of thermostable invertase.

## 1.2 Problem Statement

Use of enzymes as catalysts is limited by their stability on storage. Their stability will decreases due to changes in pH and temperature as a result of friction, osmotic pressure imposed by the environments of their use and a cumulative effect of all these factors as a function of duration of their use (Kotwal and Shankar, 2009). As temperature rises, molecules have more kinetic energy. And this will increase collision between them and the rate increase. While when pH changes, intermolecular bond break and thus changing its shape and the enzyme become effectiveness.

Enzymatic reactions may be contributed to a significant extent to these transformations. Enzymatic reactions may be desirable in some cases but are often disturb. Control of such reactions is necessary for technological improvement (Drapon, 1985) as well as preservation of quality and shelf life of foods. In industrial processes, finding a working temperature that gives high enzyme activity with good

stability represents a compromise between lower process costs and higher productivities. The selection of suitable temperature that may improve enzyme stability without considerable loss of activity is one of the main purposes of this study.

The kinetic parameters describing the catalytic mechanism of the enzyme are independent of substrate concentration. Variations in pH of medium result in changes of ionic form of the active site thus alter the shape of enzyme and lastly affect the maximum reaction rate,  $K_m$ . If an enzyme has a small value of  $K_m$ , and it achieves its maximum catalytic efficiency at low substrate concentrations. However, the value of  $K_m$  for an enzyme depends on the particular substrate. It also depends on the pH and temperature of the solution at which the reaction is carried out. Lower  $K_m$  values are generally associated with lower  $V_{max}$  values (Somero, 1978).

### 1.3 Objectives

- 1.3.1 To study the pH and thermostability of invertase by *Aspergillus niger*.
- 1.3.2 To study the enzyme kinetics of invertase.
- 1.3.3 To study the effect of sucrose concentration during incubation towards invertase activity.

## 1.4 Scope of Study

This study will be carried out in submerged fermentation using 500 mL shake flask culture. For stability study, the effect of temperature ranged for 40°C, 45°C, 50°C, 55°C and 60°C and pH ranged from 4.0, 5.0, 6.0, 7.0 and 8.0 will be investigated. For enzyme kinetic determination ( $K_m$  and  $V_{max}$ ), the initial rate concentration will be estimated at substrate concentration at range 0.2 g/L, 0.4 g/L, 0.6 g/L and 0.8 g/L. Sucrose concentration also was studied on invertase activity at 0.2 g/L, 0.4 g/L, 0.6 g/L and 0.8 g/L.

## 1.5 Rational and Significance

From this study, we can know most stable condition of invertase and from that we should be more alert and produce high quality food. Invertase is one of the essential nature enzymes uses to help us to digest sugars. It is commonly found in bee pollen and yeast sources, other than that, invertase also plays a key role not only in digestive processes, but important in human disease prevention, physical rejuvenation and anti-ageing processes. Theoretically, as we age, we have less access to this natural enzyme which resulting in a reduced ability to extract the vital nutrients from the food that we eat. It can also slow down our digestive processes, as sugars and starches are such a big part of most people diets. While some forms of sugar and carbohydrates are good for the body, they cannot be absorbed or digested well without the help of the invertase enzyme (Edward, 2011).

Other than that, study of stability of enzymes is an important aspect to consider in biotechnological processes as this can provide information on the structure of enzymes and facilitate an economical design of continuous processes in bioreactors (Jurado *et al.*, 2004). The enzyme has excellent activity at lower temperatures, but its reaction times had to be extended. To compensate for the extended reaction times, more enzymes may be used. For ideal results, invertase can be used at temperatures up to 60°C without loss of activity. But then if temperature above it, enzyme activity begins to decrease, more enzymes may be used to compensate for the loss of activity. It may be necessary to optimize applications requiring higher temperatures depending on its actual temperatures. Invertase may be used over an extended pH range with an optimum pH at 4.5.

Prolonged exposure to extreme heat and humidity can denature enzymes more rapidly and result in loss of activity. Enzymes also lose activity due to oxidation or hydrolysis. These phenomena are inherent in the enzyme, and there is little that can be done. Because of these inherent characteristics, enzymes need to be carefully stored, handled, formulated and encapsulated. It is recommended that enzymes be stored in a cool and dry environment. In addition, other conditions such as the high acidity of the gut where some enzymes may be sensitive can be overcome with enteric coatings or microencapsulated enzymes.

Anderson (2011) also stated that when it comes to enzyme formulation, stability is key point. For better enzyme, test for stability and activity, and then test again if excipients have been added. And while an enzyme's sensitivity to its environment may mean taking extra care in storage and processing, it is that same highly responsive nature that makes an enzyme finely attuned to the digestive

processes as it supports if it is properly formulated. For instance, a digestive enzyme can be prematurely activated or destroyed in the acid of the stomach, rendering it useless for nutrient absorption.

## CHAPTER 2

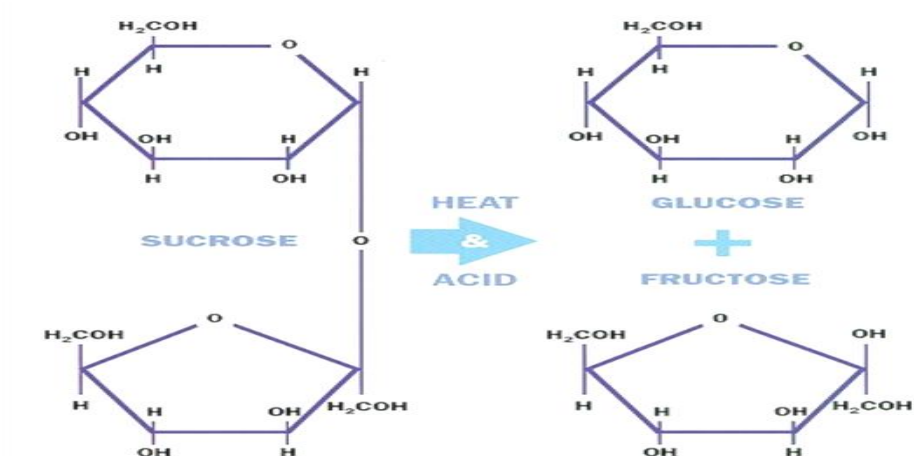
### LITERATURE REVIEW

#### 2.1 Invertase

Invertase or official name beta-fructofuranosidase is a commercially important enzyme used for the hydrolysis of sucrose. This hydrolysis of sucrose yields an equimolar mixture of glucose and fructose, known as invert sugar. Invert sugar is sometimes referred to as artificial honey since its composition and properties are nearly same. Figure 2.1 shows how sucrose hydrolysis by invert sugar. Enzymatic invert sugar is safe as it also an absolute health-friendly sweetener. So, the enzyme invertase is employed for the hydrolysis of sucrose and fructose. Nowadays, fructose is a sugar which can be consumed by diabetic patients. This because its metabolism which is insulin independent. Also, invert sugar is 28 to 30% sweeter than table sugar so the amount of sugar required for particularly degree of sweetness is also reduced. Finally, the enzymatic invert sugar does not involve the



use of any chemicals or acids unlike conventional acid-hydrolyzed invert syrup. Hence, it is completely healthy sweetener (Willkommen, 2007).



**Figure 2.1:** Hydrolysis of Sucrose Producing Glucose and Fructose

Traditionally, invertase usually produced on site by autolysing yeast cells and is sold either as a clear liquid or as a powder that can be dissolved in water. Its name refers to its ability to change the direction of optical rotation of sucrose solution as a result of hydrolysis to glucose and fructose (Whitaker, 1972). According to Verma and Dubey (2001) invertase is a group of ubiquitous enzymes with different pH optimum and subcellular localization. Invert sugar is frequently used in commercial baking and candy recipes because it keeps baked goods moist for longer periods of time. The invertase itself should be stored in the refrigerator for longevity. Optimum conditions for invertase are pH 3.0 to 6.0 and temperature 40°C to 80°C.

## 2.2 Application of Invertase

Invertase is widely used in food (confectionery) and beverage industries where fructose is preferred over sucrose because it is sweeter and does not crystallize easily. However, the use of invertase is limited because another enzyme, glucose isomerase can be used to convert glucose to fructose more inexpensively. For health and taste reasons, its use in food industry requires that invertase be highly purified. This enzyme is also used for the manufacture of artificial honey, plasticizing agents that commonly used in cosmetics, pharmaceutical industries and paper industries as well as enzyme electrodes for the detection of sucrose, high solubility, humid nature and high osmotic pressure. Invertase is used for production of inverted sugar, the valuable commercial product for the food industry in countries where the main sources of sugar is beet or cane. (Research Article, retrieved from <http://scialert.net>).

Klein *et al.* (1989) state that invertase catalyzes the cleavage of sucrose to glucose and fructose. Today, invertase is one of the most widely used enzymes in food industry, especially in preparation of jams and candies. Other than that, this invert sugar can be used in biscuits as caramelisation, enhanced flavour and better texture, bread, cake and pastries as better crust colour and softer crumb and faster yeast activation, fruit processing for better shelf life and provides better taste profile, and squashes, lemonades and instant energy drinks because glucose and fructose provide instant energy and better taste (retrieved from <http://www.ensymm.com/>). Invertase also used in pharmaceutical industry as digestive aid tablets or ready to use for cough syrups and glucose and fructose based intravenous fluids, powder milk for

infants' food, as calf feed preparation, assimilation of alcohol in fortified wines and manufactured inverted sugars as food for honeybees (Uma *et al.*, 2012).

### 2.3 *Aspergillus niger*

*Aspergillus niger* is a widely distributed filamentous fungus (Bos *et al.*, 1988). Bakri *et al.*, 2003 stated and proved that filamentous fungi are preferred for commercial enzyme production, because the level of the enzyme of these cultures is higher than those obtained from yeast and bacteria. *A. niger* has been single largest fungal source of enzymes (Subramaniyam and Vimala, 2012). *A. niger* produce colonies that are mainly composed of white or yellow that was covered by dark fungal spores. This fungus often found indoors and grows as black colonies called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and basically known as a contaminant of food. It is ubiquitous in soil and commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys chartarum*, toxic black mold species of which can infest water-damaged dwellings and cause severe symptoms in people. Black mold is ever-present in soil and decaying plant such as compost piles. Its spores are very common in air. *A. niger* also is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and in severe cases, damage to the ear canal and tympanic membrane. *A. niger* invades the outer ear canal which can cause damage to the skin it came in contact with (May, 1997).

*A. niger* is a haploid filamentous fungi and is a very essential microorganism in the field of microbiology. In addition to producing extracellular enzymes and citric acid, *A. niger* is always used for waste management and biotransformation. The fungi is most commonly found in mesophilic environments such as decaying vegetation or soil and plants (Schuster *et al.* 2002). Genome sequencing of *A. niger* is important because of its involvement in producing citric acid as well as other industrial enzymes, such as amylases, proteases, and lipases. The use of these enzymes is essential because of its importance for transformation to food enzymes. Other properties of this species include pathogens that cause the spoilage of food and production of secondary metabolites, such as aflatoxin, that are toxic. Understanding this economic importance as well as the effects it makes on the environment makes the genome sequencing of *A. niger* essential to biological applications (Takahashi *et al.*, 1991; May., 1997).

Biologically enzymes commonly isolated from any living organism such as plants, animals and microorganism. But now, microbe is preferred as sources of enzymes because of less harmful materials than plant and animal tissues. Moreover, according to Uma *et al.*, 2012, majority of enzymes used in industrial or in biotechnological applications are derived from fungi and bacteria. *A. niger* has been a very important microbe used in the field of biotechnology. Many of the industrial enzymes produced by *A. niger* such as citric acid, amylases, lipases, cellulases, xylanases and proteases, are considered GRAS (generally recognized as safe) by the United States Food and Drug Administration and is excused from the Federal Food, Drug, and Cosmetic Act food additive tolerance requirements. However, it is still important to treat this fungus very carefully to avoid formation of spore dust which can cause illness (Schuster *et al.*, 2002).

## 2.4 Effect of pH on Invertase Production

Efficient thermostable invertase was active in acidic pH. The maximum invertase production was detected by using sucrose as carbon source. At higher pH values, enzyme was higher in the medium supplemented with low sucrose concentration. It was observed that the more of fungi decreased, the greater the enzyme activity observed. It is suggested that invertase may precipitate in autolysis of fungi and could prove to be a potential source of industrial application and exploitation (Vaishali and Vrinda, 2009).

**Table 2.4:** Effect of pH on Invertase Production

pH	Optimum Activity of Invertase	References
4.6 to 6.5	pH 5.5, 0.7 IU/mL	Boddy <i>et al.</i> , 1992
3.0 to 8.0	pH 5.0, 23 IU/mL	Uma, 2010
2.0 to 8.0	pH 5.0	María and María, 1995
3.5 to 5.5	pH 4.5	Nam, 2009
2.0 to 11	pH 4.4	L'Hocine <i>et al.</i> , 2000
4.0 to 10	pH 5.0	Kaur and Sharma, 2005
4.0 to 9.0	pH 4.5	Rubio <i>et al.</i> , 1997
4.0 to 9.0	pH 5.0	Sirisansaneeyakul <i>et al.</i> , 2000

## 2.5 Effect of Temperature on Invertase Production

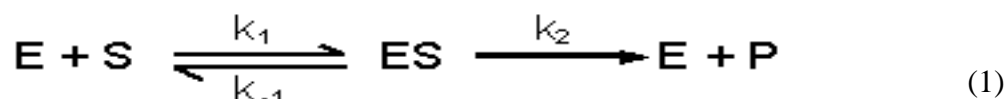
Invertase activity also effected by temperature, as the temperature increases, the rate of reaction also increase, but when it reach at certain temperature the rate of reaction drops drastically which explained by the nature of the enzymes which are proteins. All proteins with tertiary structure, like invertase, denature when exposed to high temperatures. Denatured proteins do not react as much as normal proteins, therefore lessening the reaction rate of the system (Lusung and Maningat, n.d.)

**Table 2.5:** Effect of Temperature on Invertase Production

Temperature	Optimum Activity of Invertase	References
20°C to 60°C	50°C, 40 IU/mL	Uma <i>et al.</i> , 2010
20°C to 70°C	50°C, 3.2 IU/mL	Boddy <i>et al.</i> , 1993
25°C to 75°C	60°C	María and María, 1995
30°C to 70°C	55°C,	L'Hocine <i>et al.</i> , 2000
60°C to 75°C	30°C	Rajoka and Yasmeen, 2005
20°C to 90°C	60°C	Lusung and Maningat, n.d.
50°C to 70°C	45°C	Kaur and Sharma, 2004

## 2.6 Michaelis-Menten Kinetics

From McKee and McKee (1946), one of the most useful models in the systematic investigation of enzymes rates was proposed by Leonor Michaelis and Maud Menten in 1913. The concept of enzyme-substrate complex, first enunciated by Victor Henri in 1903, is central to Michaelis-Menten kinetics. When the substrate S binds in active site of an enzyme E, an intermediate complex (ES) is formed. During transition state, the substrate is converted into product. Then the product dissociates from the enzyme. This process can summarize as:



where  $k_1$  = rate constant for ES formation

$k_{-1}$  = rate constant for ES dissociation

$k_2$  = rate constant for product formation and release from active site

The dependence of an initial rate of reaction upon the concentration of a substrate S that is present in large excess over the concentration of an enzyme or other catalyst (or reagent) E with the appearance of saturation behavior following the Michaelis–Menten equation:

$$v = \frac{V_{\max} [S]}{[S] + K_m} \quad (2)$$

where  $v$  is the observed initial rate

$V_{\max}$  is its maximum velocity

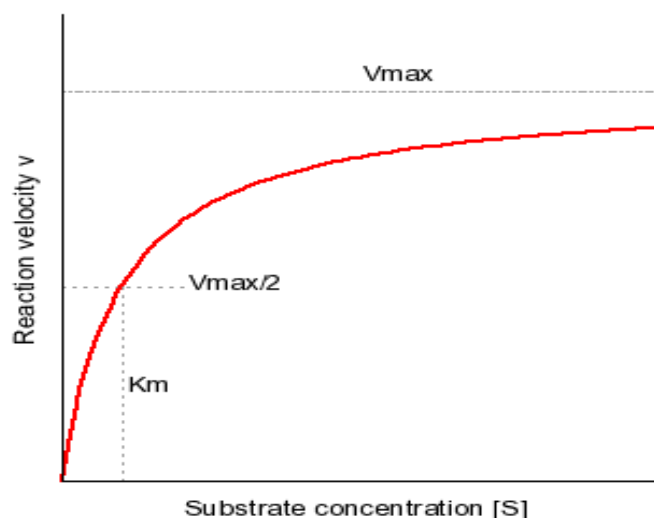
$K_m$  the substrate concentration when the enzyme has half-maximal velocity

The Michaelis constant  $K_m$  is defined as the substrate concentration at half the maximum velocity  $V_{max}$ . Michaelis developed the following expression for the reaction velocity in terms of this constant and the substrate concentration. Michaelis constants have been determined for many of the commonly used enzymes. The size of  $K_m$  can tell us several things about a particular enzyme.

1. A small  $K_m$  indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.

2. A large  $K_m$  indicates the need for high substrate concentrations to achieve maximum reaction velocity.

3. The substrate with the lowest  $K_m$  upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes. (Retrieved from <http://www.worthington-biochem.com/introbiochem/substrateconc.html>).



**Figure 2.6.1:** Michaelis-Menten Model

Source: Shuler and Kargi 2002